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TRANSFORMING EMBRYOGENIC CELL LINES OF *GLADIOLUS* WITH EITHER A *BAR-UIDA* FUSION GENE OR COBOMBARDMENT

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Summary

Embryogenic cell lines of *Gladiolus* were bombarded with the *bar-uidA* fusion gene under the cauliflower mosaic virus (CaMV) 35S promoter (pDM327) or cobombarded with *uidA* under the CaMV 35S promoter (pBCG) and *bar* under the CaMV 35S promoter (pDM307). Over 500 cell lines were isolated for either the fusion gene or cobombarded cells following selection on Murashige and Skoog's medium supplemented with 2 mg l^{-1} (9 μM) 2,4-dichlorophenoxyacetic acid and 6 mg l^{-1} phosphinothricin. The optimum DNA concentration for isolating stable transformants was one-tenth that for optimal isolation of lines with *gus* expression, and three times as many cell lines were isolated following cobombardment as compared to bombardment with the *bar-uidA* fusion gene. Three times as many cell lines (72% of the cell lines) containing the *bar-uidA* fusion gene expressed *gus* as compared to cobombarded cell lines (23%) following histological staining. *Gus* expression ceased after 1 yr in culture for 5% of the cell lines containing the fusion gene and 3% of the cobombarded cell lines. The bifunctionality and utility of the *bar-uidA* fusion gene were demonstrated, accompanied by enhanced *gus* expression.

Key words: monocot transformation; reporter gene; selectable marker gene; β-glucuronidase.

Introduction

A bifunctional *bar-uidA* translational fusion gene was developed to combine the benefits of the *bar* gene as a selectable marker and the *uidA* gene for monitoring transformation efficiency and transgene expression. Selectable marker genes are used to discriminate between transgenic and nontransgenic cells in systems that display low transformation efficiencies. Resistance to phosphinothricin (PPT)-based herbicides using the *bar* gene from *Streptomyces hygroscopicus* has become the method of choice for the selection of fertile transgenic cereals (Casas et al., 1993; Christou et al., 1991; Fromm et al., 1990; Gordon-Kamm et al., 1990; Vasil et al., 1992; Wan and Lemaux, 1994; Weeks et al., 1993). PPT is a glutamate analog that inhibits glutamine synthase, leading to accumulation of ammonia and cell death. The *bar* gene codes for phosphinothricin-*N*-acetyltransferase (PAT), which inactivates PPT by acetylation.

An equally important component of any plant transformation scheme involves the use of reporter genes for optimizing gene delivery, monitoring selection efficiency and evaluating gene expression. The β-glucuronidase (*gus*) gene (Jefferson and Wilson, 1991) encoded by the *uidA* locus of *Escherichia coli* has been the most popular reporter gene used in plant transformation (McElroy and Brettell, 1994). *Gus* catalyzes the hydrolysis of a wide range of fluorimetric and histochemical *Gus* substrates. GUS enzyme activity can be easily and sensitively assayed in plants, the expression of *gus* gene fusions can be quantified by fluorimetric assay and histochemical analysis can be used to localize gene activity in transgenic tissues.

Despite its widespread use, not all species of plants express *gus*. Studies have shown that while some transgenes, such as *bar*, will continue to be expressed over a long period of time and following its transmission to progeny after meiosis, others, such as *gus*, will frequently cease expression with time (Spencer et al., 1992; Register et al., 1994). Gene fusions have been used in *E. coli* to alter the properties of a recombinant protein, resulting in both increased levels and solubility of the protein product (Chopra et al., 1994; La Vallie and McCoy, 1995). Transgenic plants containing fusion genes have shown that a fusion protein will function in plants; for example the fusion of *uidA* with the light-inducible GOS5 gene which resulted in *gus* expression in three monocot species (Hensgens et al., 1993).

It was anticipated that fusion of the *bar* gene with *uidA* gene would stabilize the *uidA* transcript, resulting in an increased number of cell lines recovered that expressed *gus*, and that *gus* expression would continue for a longer period with a fusion gene as compared to *gus* expression resulting from cobombardment. This study confirmed that the *bar-uidA* fusion gene increased GUS enzyme activity five- to 16-fold following transient transformation of wheat and maize protoplasts. In addition, there was a threefold

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increase in the number of cell lines recovered that expressed *gus* following stable transformation of embryogenic *Gladiolus* cells with the *bar-uidA* fusion gene as compared to cobombarded cell lines.

The *bar-uidA* fusion gene was applied to *Gladiolus*, a monocotyledonous flower that has been previously transformed using the PAT gene (Kamo et al., 1995). *Gladiolus* ranks fifth in terms of the number of stems shipped worldwide (U.S. Dept. Of Agriculture, 1994), but its production is diminished by viral and fungal pathogens, making *Gladiolus* a commercially important floral crop that would benefit tremendously from transgene-mediated disease resistance. Transgenic plants of *Gladiolus* containing the *bar-uidA* fusion gene under various promoters have been developed, and these plants are being characterized for tissue-specific expression by the promoters (Kamo and Blowers, 1999; Kamo et al., 2000).

This study required the use of an embryogenic, nonregenerable cell line that consists of small cell clusters with a high frequency of transformation, making it possible to isolate 1000 cell lines for a significant comparison between transformation and expression frequencies using either the *bar-uidA* fusion gene or cobombardment. A regenerable cell line of *Gladiolus* consists of large (3–5 mm) cell clusters and has a much lower frequency of transformation.

Materials and Methods

Construction of a bar-uidA *translational fusion gene.* All enzymatic DNA manipulations, including subcloning strategies, Klenow DNA polymerase fill-in reactions and double-stranded sequencing of construct junction regions, were performed following standard protocols (Sambrook et al., 1989) using modifications recommended by the enzyme manufacturers.

The *bar* coding region (minus the translation termination codon) was isolated as a 0.6 kbp *Eco* RI-*Bgl* II restriction fragment from pIJ4104 (Murakami et al., 1986), and this was cloned between the *Eco* RI and *Bam* HI restriction sites of the *uidA-nos*-containing construct pDM201.3 (McElroy et al., 1995) to create the *bar-uidA-nos* translational fusion construct pDM323. The translation termination codon of the *bar* coding region in construct pIJ4104 was deleted by digesting the plasmid with *Bgl* II and *Bam* HI and self-ligating the linearized DNA to create the construct pDM310. The *bar* coding region was isolated as a 0.6 kbp *Eco* RI-*Pst* I restriction fragment from pDM310, and this was cloned between the *Eco* RI and *Pst* I restriction sites of the *uidA-nos*-containing construct pDM201.3 to create the *bar-uidA-nos* translational fusion construct pDM333.

The cauliflower mosaic virus (CaMV) 35S promoter was isolated as a 0.8 kbp *Hind* III-*Eco* RI restriction fragment from the 35S-*bar-nos*-containing construct pDM307 (Cao et al., 1992), and this was cloned between the *Hind* III and *Eco* RI restriction sites of pDM323 and pDM333 to create the *35S-bar-uidA-nos* translational fusion constructs pDM327 and pDM337, respectively.

PAT assays. PAT activity in mid-log phase *E. coli* DH5α cells (Spencer et al., 1990), tobacco (*Nicotiana plumbaginifolia*), wheat and rice protoplasts 48 h after electroporation (Chamberlain et al., 1994) was analyzed using a procedure of De Block et al. (1987). ^{14}C-Acetylated PPT production was visualized by autoradiography and quantified by Phospho-Imager analysis.

Tissue culture. The wheat (cv. L1), maize (Black Mexican Sweet), tobacco suspension cultures and isolation of protoplasts from these cell suspensions were as previously described (Chamberlain et al., 1994; McElroy et al., 1991).

Gladiolus cv. Jenny Lee suspension cells were cultured in MS (Murashige and Skoog, 1962) basal salts medium containing 3% (w/v) sucrose and the following (mg l^{-1}): glycine, 1.0; thiamine, 1.0; pyridoxine, 0.5; nicotinic acid, 0.5; and 2,4-dichlorophenoxyacetic acid (2,4-D), 2.0 (9 μ*M*). Cells were grown in 125 ml flasks containing 20 ml medium and subcultured every week at a 1:2 dilution to fresh medium and grown in the dark at 26°C on a gyratory shaker (120 rpm).

Suspension cells were placed on selection medium 1 wk after biolistic bombardment. Selection medium consisted of MS basal salts medium as above, containing 2.0 mg l^{-1} (9 μ*M*) 2,4-D and 6 mg l^{-1} PPT (AgrEvo Co., Somerville, NJ) solidified with 2 g l^{-1} Gelrite (Sigma Chemical Co., St. Louis, MO), and cells were transferred every 3 wk to fresh medium. Callus cells were grown at 26°C in the dark.

Biolistics. The PDS-1000/He system (BioRad, Richmond, CA) was used for delivery of 1.0 μm gold particles to suspension cells. Gold particles were coated with plasmid DNA according to the method of Sanford et al. (1993). Ten μg gold (10 μl) was coated with 2 μg DNA (1 μg μl^{-1} or the desired dilution) to which was added 10 μl 2.5 *M* $CaCl_2$ and 4 μl 100 m*M* spermidine in a total volume of 26 μl for bombarding a single plate of suspension cells. Plasmid DNA was isolated using alkaline lysis followed by cesium chloride gradient purification (Maniatis et al., 1982). The particle gun had a 1 cm gap and flying membrane distance. Suspension cells were cultured for 3 h in liquid MS medium supplemented with 0.125 *M* mannitol prior to being plated on Whatman no. 4 filter paper for bombardment at 8.3 mPa (1200 psi) once per plate at a 12 cm target distance. Each experiment consisted of three plates for each DNA concentration, and the experiment was repeated six times. At least 100 cell lines, each representing an independent transformant, were isolated for each DNA concentration of pDM327 or cobombarded plasmid DNAs.

Gus *assay.* Histochemical staining of cells was carried out according to the procedure of Jefferson et al. (1987).

Genomic DNA analysis. Genomic DNA was isolated from suspension cells according to the method of Dellaporta et al. (1983). DNA (20 μg) was digested with *Hind* III and then subjected to electrophoresis in a 0.7% agarose gel in TBE buffer (89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA, pH 8.0) at 75 V, 50 mA for approximately 4 h. The DNA was transferred to Nytran nylon membrane (Schleicher and Schuell, Keene, NY) by capillary action (Maniatis et al., 1982). The probe used for hybridization was pDM327 digested with *Sma* I and *Sst* I to release the *uidA* gene; this was followed by gel purification and labeling by random primer [α-^{32}P]dCTP using the Megaprime Kit (Amersham, Arlington Heights, IL). Blots were incubated in prehybridization buffer for 2 h at 42°C followed by hybridization buffer and probe at 60°C for 16 h Maniatis et al., 1982). Three washes in 6× SSC, 1% sodium dodecyl sulfate for 1 min each at 65°C, and two washes in 2× SSC for 5 min each at 26°C were done, followed by exposure of the blot at −70°C with an intensifying screen.

Results and Discussion

Construction of bar-uidA *translational fusion genes.* *Bar-uidA* genes with both short (pDM327) and long (pDM337) junction sequences between the two coding regions were constructed as shown by the restriction map (Fig. 1A). The sequence and predicted translation products of both the short and long *bar-uidA* junction regions are shown in Fig. 1B.

PAT activity of the bar-uidA *translational fusion genes.* The *bar* gene codes for PAT, which acetylates PPT to yield the inactive product *N*-acetyl-PPT. The CaMV 35S promoter is known to function in bacterial cells (Assaad and Singer, 1990), making it possible to chromatographically assay the PPT acetylation activity of the two 35S-*bar-uidA* translational fusion constructs in *E. coli* cells using Basta® (a herbicide containing PPT) and ^{14}C-acetyl CoA as substrates. The PAT activity of the 35S-*bar-uidA* genes relative to that of the 35S-*bar* gene was determined (Fig. 2). The construct pIJ4104, containing the *bar* gene under the control of the inducible *lacZ* promoter from *E. coli*, was used as a positive control of *E. coli* PAT activity. Tobacco material expressing a CaMV 35S-*bar* gene was used as a positive control for 35S-*bar* gene expression. In *E. coli* it was found that PAT activity was between 20% (pDM337) and 35% (pDM327) that of the 35S-*bar* gene (pDM307).

Transient gus *activity of the* bar-uidA *translational fusion genes.* *Gus* enzyme activity from the various *bar-uidA* translational fusion genes was quantified by fluorimetric assay of electroporated

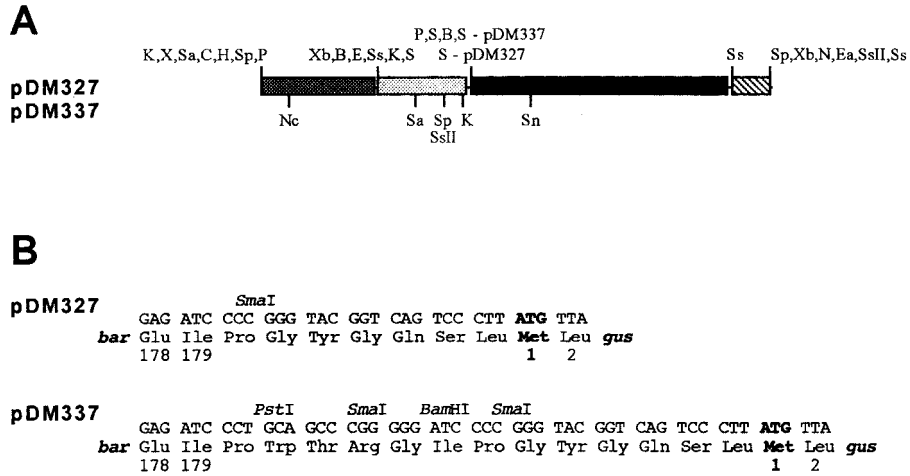

Fig. 1. A. Restriction map of the 35S-*bar-uidA-nos* translational fusion genes pDM327 and pDM337. The different regions of the *bar-uidA* fusion genes are indicated as follows: heavy stippled box, CaMV 35S promoter; light stippled box, *bar* coding region; filled box, *uidA* coding region; hatched box, 3′ noncoding region of the nopaline synthase gene. The restriction sites are abbreviated as follows: B, *Bam* HI; C, *Cla* I; E, *Eco* RI; Ea, *Eag* I; H, *Hind* III; K, *Kpn* I; N, *Not* I; Nc, *Nco* I; P, *Pst* I; S, *Sma* I; Sa, *Sal* I; Sn, *Sna* BI; Ss, *Sst* I; I; Ss II, *Sst* II; Sp, *Spe* I; X, *Xho* I; Xb, *Xba* I. B. Nucleotide sequence of the junction region of the *bar-uidA* fusion genes. Restriction sites used in the construction of the various *bar-uidA* fusion genes are indicated. The predicted amino acid sequences of the various *bar-uidA* junction regions are indicated below their respective codons. The last two codons of the *bar* coding region and the first two codons of the *uidA* coding region are numbered, with the *uidA* translation initiation codon indicated in bold.

wheat, maize and tobacco protoplasts (Table 1). Both of the 35S-*bar-uidA* fusion genes showed five- to 16-fold higher GUS activities than the 35S-*uidA* gene of plasmid pBCG in the cereal monocots wheat and maize.

Selection of transformed Gladiolus *cell lines.* Transformed *Gladiolus* cell lines were evident 1 mo. after bombardment. The maximum number of cell lines was recovered from cobombardments with 10 ng μl^{-1} plasmid DNA or with either 10 or 100 ng μl^{-1} of the *bar-uidA* fusion gene pDM327 (Fig. 3).

DNA blots hybridized with *uidA* showed a trend toward lower copy number of pDM327 as the DNA concentration used to coat gold particles decreased (Fig. 4). Five of the six transformants that resulted from bombardment with 0.1 ng μl^{-1} DNA contained one to two copies of the transgene, and one transformant contained four copies (Fig. 4A). All six transformants that resulted from bombardment with 1 ng μl^{-1} DNA contained one to two copies of the transgene (Fig. 4B). In comparison, the six transformants resulting from bombardment with 1000 ng μl^{-1} DNA contained four to nine copies of the transgene (Fig. 4C).

There were more cell lines of *Gladiolus* recovered using co-transformation with pDM307 and pBCG as compared to using the *bar-uidA* fusion gene pDM327 at all DNA concentrations, ranging from 0.1–1000 ng μl^{-1}, that were used for coating gold particles (Fig. 3). These results suggest that *bar* expression is more effective when *bar* is alone rather than fused to *uidA*. This result is in agreement with the assay of PAT activity in *E. coli* which showed that PAT activity with *bar-uidA* fusion was 35% of the 35S-*bar* gene (pDM307) (Fig. 2).

Gus *expression by transformed cell lines.* The percentage of cell

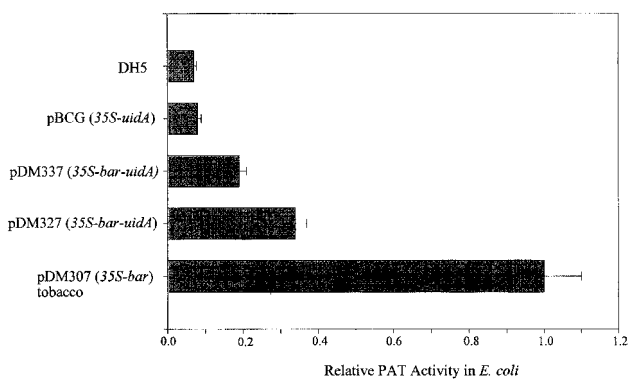

Fig. 2. Analysis of the PAT activity in extracts of mid-log phase *E. coli* DH5α (containing *bar* and *bar-uidA* fusion genes. The *bar* gene encodes PAT that acetylates PPT to yield the inactive product *N*-acetyl-PPT, using acetyl-CoA as the acetyl donor. Bar graph showing PAT activity of the 35S-*bar-uidA* genes relative to that of the 35S-*bar* gene (pDM307). The construct pIJ4104 containing the *lacZ-bar* gene was used as a positive control for 35S-*bar* gene expression, and three independent PAT assays were performed.

TABLE 1

GUS ACTIVITIES OF *BAR-UIDA* FUSION GENES IN ELECTROPORATED WHEAT, MAIZE AND TOBACCO PROTOPLASTS

Plasmid		*Gus* specific activity[a] (pmol MU mg protein^{-1} h^{-1}) Wheat	Maize	Tobacco
pBCG	35S-*uidA-nos*	162ÿ± 18	4020ÿ± 510	105ÿ± 22
pDM307	35S-*bar-nos*	ND	ND	ND
pDM327	35S-*bar-uidA-nos*	2,556ÿ± 204	21,180ÿ± 1,284	193ÿ± 17
pDM337	35S-*bar-uidA-nos*	1,980ÿ± 78	28,571ÿ± 1,002	177ÿ± 19

[a] Results given as mean±standard error (S.E.) of the mean with *gus* specific activity after correction for background. ND = not detected above background. Results are from three independent experiments assayed 3 d after transformation by electroporation.

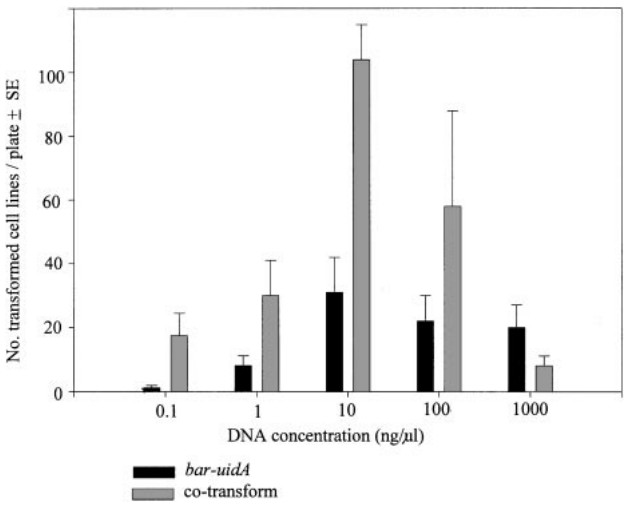

Fig. 3. The number of stably transformed cell lines of *Gladiolus* recovered following bombardment with various DNA concentrations of the *bar-uidA* fusion gene pDM327 or from cobombardment using pDM307 and pBCG.

lines (72%) that expressed *gus* was higher for cell lines containing the *bar-uidA* fusion gene pDM327 than for cobombarded cell lines (23%) (Fig. 5). Coating gold particles with either 100 or 1000 ng μl^{-1} pDM327 resulted in the maximum number of cell lines recovered that expressed *gus* from the fusion gene. The maximum number of cell lines that expressed *gus* resulted following cobombardment with a total of 100 ng μl^{-1} pBCG and pDM307.

Copy number has been shown to be one of many factors that may affect gene expression, and the effect appears to differ with each plant species. Transgenic rice resulting from cotransformation showed higher expression when there were fewer copies of the *uidA* transgene (Christou, 1997). In comparison, transgenic wheat was shown to have increased levels of expression for both the *uidA* and *bar* genes that correlated with higher copy number (Stoger et al., 1998). In this study with *Gladiolus*, the number of cell lines that expressed *gus* increased with increasing DNA concentration used for bombardment, and the DNA blots showed that DNA concentration used for bombardment affected copy number; therefore it appears that there was a negative correlation between copy number and *gus* expression.

The percentage of cell lines that ceased *gus* expression after 1 yr in culture was similar for cell lines recovered following transformation with the *bar-uidA* fusion gene (5%) or cobombardment (3%) (Table 2). It was anticipated that more cobombarded cell lines rather than *bar-uidA* fusion cell lines would stop expressing *gus* after 1 yr because of the possibility of gene silencing resulting from the presence of two plasmids that both contain the 35S promoter; however, this did not occur after 1 yr in culture.

Conclusion

The bifunctionality of the *bar-uidA* coding region of the translational fusion gene pDM327 has been demonstrated. The bifunctional gene allowed us to benefit from the utility of the *bar*

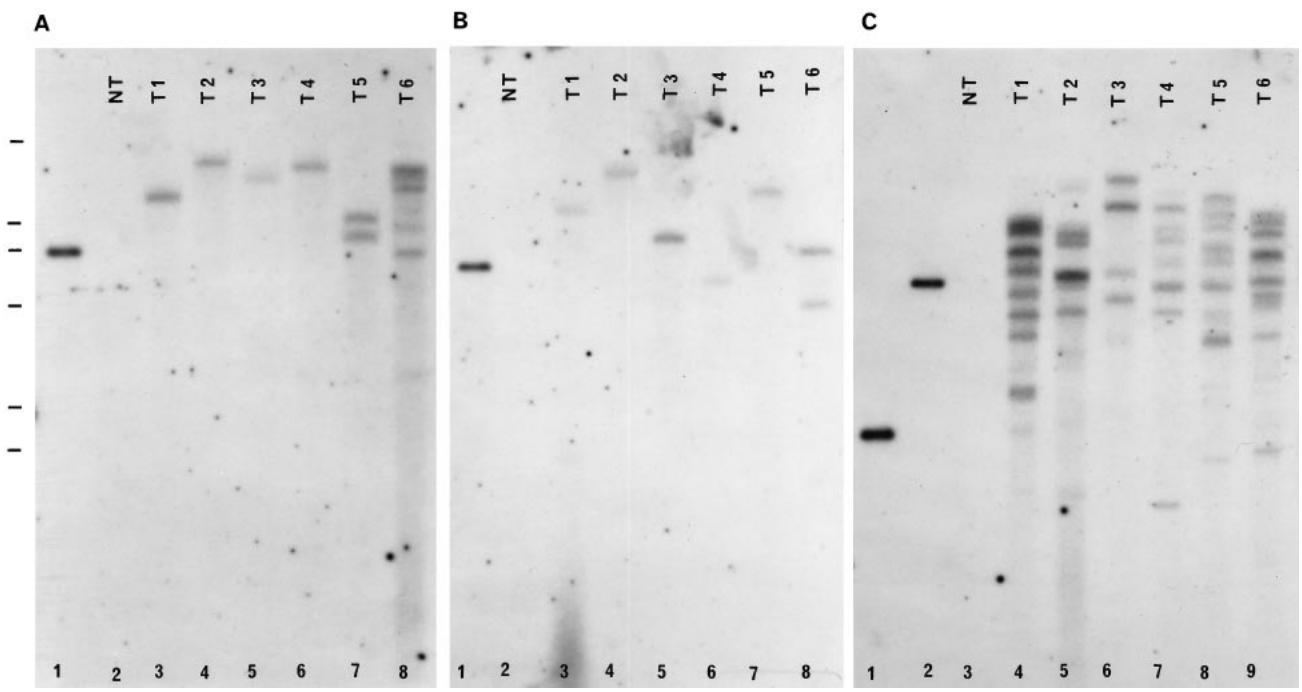

Fig. 4. DNA blot of genomic DNA isolated from cells of *Gladiolus* lines probed with pDM327 digested with *Sma* I and *Sst* I to release the *uidA* gene. The pDM327 concentration (ng μl^{-1}) used for coating gold particles was: (A) 0.1; (B) 1.0; (C) 1000. *Lanes* 1 [(A) and (B)] and 2 (C) contain 100 pg pDM327 digested with *Hind* III. *Lane* 1 (C) contains pDM327 digested with *Sst* I to release 50 pg of the *bar-uidA* insert. Genomic DNA (20 µg) was digested with *Hind* III for nontransformed (NT) [(A) and (B) *lane* 2; (C) *lane* 3)] or six transformants (T1–T6) [(A) and (B) *lanes* 3–8; (C) *lanes* 4–9)]. In (A) the molecular weight markers are 23.1, 9.4, 6.7, 4.4, 2.3 and 2.0 kbp as shown from top to bottom.

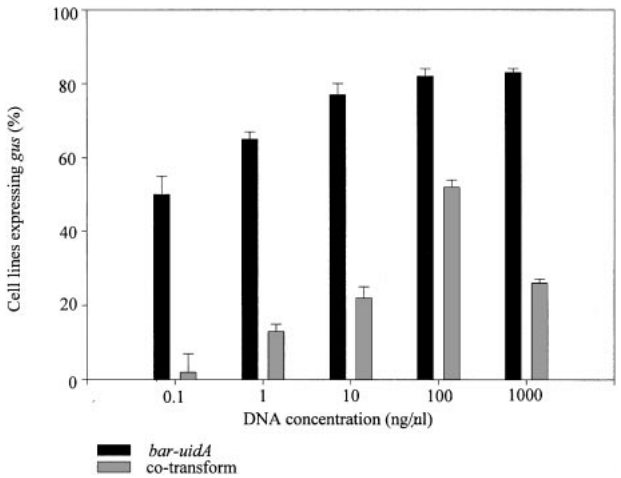

Fig. 5. The percentage of cell lines of *Gladiolus* expressing *gus* following bombardment with various DNA concentrations of the *bar-uidA* fusion gene pDM327 or cobombardment with both pDM307 and pBCG. Each experiment consisted of three plates of cells bombarded with each DNA concentration for either the *bar-uidA* fusion gene or for cobombardment, and the experiment was repeated six times.

gene as a selectable marker in monocot transformation and for visualizing transgene expression with the *uidA* gene. The fusion gene pDM327 resulted in a threefold increase in the number of cell lines of *Gladiolus* that expressed *gus*, as compared to cobombarded cell lines, indicating its usefulness when isolating stable transformants that express *gus*. The concentration of DNA used for coating the gold particles used for bombardment was shown to affect copy number of the transgene in the transgenic cell lines, the number of transformed cell lines isolated and the percentage of cell lines that showed *gus* expression.

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TABLE 2

CELL LINES OF *GLADIOLUS* (% TOTAL) THAT CEASED *GUS* EXPRESSION AFTER 1 YR IN CULTURE[a]

DNA concentration (ng μl^{-1})	*bar-uidA* Fusion gene	Cobombardment
0.1	2	1
1	6	0
10	7	7
100	4	3
1000	4	5

[a] At least 100 cell lines were isolated for each DNA concentration used to coat gold particles for bombardment. *Gus* expression was determined by histochemical staining of cells.

References

Assaad, F. F.; Singer, E. R. Cauliflower mosaic virus 35S promoter activity in *Escherichia coli*. Mol. Gen. Genet. 223:517–520; 1990.

Cao, J.; Duan, X.; McElroy, D.; Wu, R. Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Rep. 11:586–591; 1992.

Casas, A. M.; Kononowicz, A. K.; Zehr, U. B.; Tomes, D. T.; Axell, J. D.; Butler, L. G.; Bressan, R. A.; Hasegawa, P. M. Transgenic sorghum plants via microprojectile bombardment. Proc. Natl. Acad. Sci. USA 90:11212–11216; 1993.

Chamberlain, D. A.; Brettell, R. I. S.; Last, D. I.; Witrzens, B.; McElroy, D.; Dolferus, R.; Dennis, E. S. The use of a highly active Emu promoter with antibiotic and herbicide resistance genes for the selection of transgenic wheat callus and rice plants. Aust. J. Plant Physiol. 21:95–112; 1994.

Chopra, A. K.; Brasier, A. R.; Das, M.; Xu, X. J.; Peterson, J. W. Improved synthesis of *Salmonella typhimurium* enterotoxin using gene fusion expression systems. Gene 144:81–85; 1994.

Christou, P. Rice transformation. Plant Mol. Biol. 35:197–203; 1997.

Christou, P.; Ford, T.; Kofron, M. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology 9:957–962; 1991.

De Block, M.; Botterman, J.; Vandewiele, M.; Dockx, J.; Thoen, C.; Gosselle, V.; Rao Movva, N.; Thompson, C.; Van Montagu, M.; Leemans, J. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. 6:2513–2518; 1987.

Dellaporta, S. J.; Wood, J.; Hicks, J. A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1:19–21; 1983.

Fromm, M. E.; Morrish, F.; Armstrong, C.; Williams, R.; Thomas, J.; Klein, T. M. Inheritance and expression of chimaeric genes in the progeny of transgenic maize plants. Bio/Technology 8:833–844; 1990.

Gordon-Kamm, W. J.; Spencer, T. M.; Mangano, M. L.; Adams, T. R.; Daines, R. J.; Start, W. G.; O'Brien, J. V.; Chambers, S. A.; Adams, W. R.; Willetts, N. G.; Rice, T. B.; Mackey, C. J.; Krueger, R. W.; Kausch, A. P.; Lemaux, P. G. Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618; 1990.

Hensgens, L. A.; de Bakker, E. P.; van Os-Ruygrok, E. P.; Rueb, S.; van de Mark, F.; van der Maas, H. M.; van der Veen, S.; Kooman-Gersmann, M.; Hart, L. T.; Schilperoort, R. A. Transient and stable expression of *gusA* fusion with rice genes in rice, barley and perennial ryegrass. Plant Mol. Biol. 23:643–669; 1993.

Jefferson, R. A.; Kavanagh, T. A.; Bevan, M. W. GUS Fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901–3907; 1987.

Jefferson, R. A.; Wilson, K. J. The GUS gene fusion system. In: Gelvin, S. B.; Schilperoort, R. A.; Verma, D. P. S. eds. Plant molecular biology manual. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1991; B 14/1–B 14/33.

Kamo, K.; Blowers, A. Tissue specificity and expression level of *gusA* under *rolD*, mannopine synthase and translation elongation factor 1 subunit α promoters in transgenic *Gladiolus* plants. Plant Cell Rep. 18:809–815; 1999.

Kamo, K.; Blowers, A.; McElroy, D. Effect of the cauliflower mosaic virus 35S, actin, and ubiquitin promoters on *uidA* expression from a *bar-uidA* fusion gene in transgenic *Gladiolus* plants. In Vitro Cell. Dev. Biol.–Plant 36:13–20; 2000.

Kamo, K.; Blowers, A.; Smith, F.; Van Eck, J.; Lawson, R. Stable transformation of *Gladiolus* using suspension cells and callus. J. Am. Soc. Hortic. Sci. 120:347–352; 1995.

La Vallie, E. R.; McCoy, J. M. Gene fusion expression systems in *Escherichia coli*. Curr. Opin. Biotechnol. 6:501–506; 1995.

Maniatis, T., Fritsch, E.F., Sambrook, J., Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press; 1982.

McElroy, D.; Blowers, A. D.; Jenes, B.; Wu, R. Construction of expression vectors based on the rice actin 1 (*Act1*) 5′ region for use in monocot transformation. Mol. Gen. Genet. 231:150–160; 1991.

McElroy, D.; Brettell, R. I. S. Foreign gene expression in transgenic cereals: progress and pitfalls. TIBTECH 12:62–66; 1994.

McElroy, D.; Chamberlain, D. A.; Moon, E.; Wilson, K. J. Development of *gusA* reporter gene constructs for cereal transformation: availability of plant transformation vectors from the CAMBIA Molecular Genetic Resource Service. Mol. Breed 1:27–37; 1995.

Murakami, T.; Anzai, H.; Imai, S.; Satoh, A.; Nagaoka, K.; Thompson, C. J. The bialaphos biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning and characterization of the gene cluster. Mol. Gen. Genet. 205:42–50; 1986.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473–497; 1962.

Register, J. C.III; Peterson, D. J.; Bell, P. J.; Bullock, W. P.; Evans, I. J.; Frame, B.; Greenland, A. J.; Higgs, N. S.; Jepson, I.; Jiao, S.; Lewnau, C. J.; Sillick, J. M.; Wilson, H. M. Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. Plant Mol. Biol. 25:951–961; 1994.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. Molecular cloning: a laboratory manual. 2; Cold Spring Harbor Laboratory Press; 1989.

Sanford, J. C.; Smith, F. D.; Russell, J. A. Optimizing the biolistic process for different biological applications. Methods Enzymol. 217:483–510; 1993.

Spencer, T. M.; O'Brien, J. V.; Start, W. G.; Adams, T. R.; Gordon-Kamm, W. J.; Lemaux, P. G. Segregation of transgenes in maize. Plant Mol. Biol. 18:201–210; 1992.

Spencer, T. M.; Gordon-Kamm, W. J.; Daines, R. J.; Start, W. G.; Lemaux, P. G. Bialaphos selection of stable transformants from maize cell culture. Theor. Appl. Genet. 79:625–631; 1990.

Stoger, E.; Williams, S.; Keen, D.; Christou, P. Molecular characteristics of transgenic wheat and the effect on transgene expression. Transgenic Res. 7:463–471; 1998.

U.S. Department of Agriculture. Fresh fruit, vegetables and ornamental crops. Report No. WS-04-94; 1994.

Vasil, V.; Castillo, A. M.; Fromm, M. E.; Vasil, I. K. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technology 10:667–674; 1992.

Wan, Y.; Lemaux, P. G. Generation of large numbers of independently transformed fertile barley plants. Plant Physiol. 104:37–48; 1994.

Weeks, T.; Anderson, O. D.; Blechl, A. E. Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). Plant Physiol. 102:1077–1084; 1993.